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The Purification and Characterization of Mannosidase IA from Rat Liver Golgi Membranes*

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Rat liver Golgi membranes contain two α1,2-specific mannosidases (IA and IB) (Tulsiani, D. R. P., Hubbard, S. C., Robbins, P. W., and Touster, O. (1982) *J. Biol. Chem.* 257, 3660–3668). Mannosidase IA has now been purified to apparent homogeneity by detergent extraction and $(NH_4)_2SO_4$ precipitation, followed by Sephacryl S-300, ion-exchange, and hydroxylapatite chromatography. The enzyme was homogeneous by nondenaturing polyacrylamide gel electrophoresis with different gel concentrations, and Ferguson plot analysis indicated an M_r of 230,000 for the native enzyme. Although electrophoresis under denaturing conditions generally gave a subunit M_r of 57,000, electrophoresis of less than 1 μg of protein yielded a faint doublet of M_r 57,000 and 58,000. Thus, the enzyme appears to be a tetramer with four very similar subunits. The enzyme bound to concanavalin A-Sepharose 4B only when it was kept in contact with the lectin for 16 h. Endoglycosidase H treatment resulted in loss of its binding to the lectin, without leading to a detectable change in the size of the enzyme subunit. On electrophoretic gels, the enzyme gave a faint positive stain with periodic acid-Schiff's base. The enzyme contained about 0.9% hexose by direct analysis. It did not bind to affinity resins specific for neuraminic acid, galactose, or *N*-acetylglucosamine. All these studies suggest that the enzyme is a glycoprotein containing only one or two clusters of high mannose oligosaccharide. Mannosidase IA is active toward oligosaccharides containing α1,2-linked mannosyl residues. [^{3}H]$Man_9GlcNAc$, [^{3}H]$Man_8GlcNAc$, [^{3}H]$Man_7GlcNAc$, and [^{3}H]$Man_6GlcNAc$ are good substrates. $Man_9GlcNAc$, the best substrate, yields Man_8, Man_7, and Man_6 derivatives with structures suggesting that the sequence of release of mannose residues is rather specific. Immunoprecipitation studies using polyclonal antibody (IgG) prepared against homogeneous mannosidase IA cross-reacted with mannosidase IB, a result suggesting that these two enzymes share antigenic determinants. However, no cross-reactivity was observed with rat liver cytosolic and lysosomal α-D-mannosidases or with Golgi mannosidase II.

The pathway for the synthesis of glycoproteins containing asparagine-linked oligosaccharides involves the transfer of $Glc_3Man_9GlcNAc_2$ from a lipid carrier to polypeptides in the rough endoplasmic reticulum (1–4). The 3 glucose residues are removed by two glucosidases (I and II) in the endoplasmic reticulum. The resulting high mannose oligosaccharides are converted into complex-type mainly in the Golgi apparatus. This conversion requires the removal of 6 mannose residues and addition of sugars such as *N*-acetylglucosamine, galactose, fucose, and sialic acid (5, 6).

Recent evidence indicates that rat liver contains four processing mannosidases (7–11). Liver endoplasmic reticulum α-D-mannosidase (7, 8) and Golgi mannosidases IA and IB (9, 11) are able to cleave α1,2-linked mannosyl residues from asparagine-linked $Man_9GlcNAc_2$ to form $Man_5GlcNAc_2$. This oligosaccharide is modified by *N*-acetylglucosaminyltransferase I to form $GlcNAcMan_5GlcNAc_2$ (12, 13). Subsequently, Golgi mannosidase II removes terminal α1,3- and α1,6-mannosyl residues to yield $GlcNAcMan_3GlcNAc_2$ (10, 11), which is further modified by the addition of other sugars (5, 6).

Rat liver Golgi mannosidase I activity was separated into two forms by Tabas and Kornfeld (9), one of which was partially purified and characterized by these investigators. Subsequently, we reported partial purification and characterization of two distinct α1,2-specific activities (IA and IB) from rat liver Golgi membranes (11). In this study, we describe the purification of α1,2-specific Golgi mannosidase IA to a homogeneous state. Polyclonal antibody prepared against the homogeneous enzyme was tested for its ability to cross-react with other α-mannosidases. A preliminary report of this work has been presented (14).

EXPERIMENTAL PROCEDURES

Materials—Male Wistar rats (125–150 g) were from Harlan Industries, Inc. 1-Deoxymannojirimycin was kindly provided by Dr. Gunter Legler (University of Cologne). Oligosaccharides, uniformly labeled with [^{3}H]mannose, namely [^{3}H]$Man_9GlcNAc$, [^{3}H]$Man_8GlcNAc$, [^{3}H]$Man_7GlcNAc$, [^{3}H]$Man_6GlcNAc$, and [^{3}H]$Man_5GlcNAc$, were isolated from a single preparation as described (15). [^{3}H]Mannose-labeled glycopeptides containing hybrid oligosaccharide ($NeuAcGalGlcNAcMan_5GlcNAc_2Asn$) and complex oligosaccharides ($(NeuAcGalGlcNAc)_{2-4}Man_3GlcNAc_2Asn$) were prepared by Pronase digestion of labeled cellular glycoproteins as described (15). *Staphylococcus aureus* cells (IgGSorb) were obtained from the Enzyme Center, Inc. Bio-Gel P-2 (200–400 mesh), Bio-Gel P-4 (−400 mesh), and hydroxylapatite (Bio-Gel HT) were from Bio-Rad. Cellulose phosphate (Sigma), microgranular DE52 (Whatman), and Sephacryl S-300 (Pharmacia LKB Biotechnology Inc.) were used as recommended by the manufacturers. Salt-washed rat liver Golgi membranes were prepared as described (10). Rat liver lysosomal α-D-mannosidase was purified as described (16). Homogeneous Golgi mannosidase II and partially purified Golgi mannosidase IB were prepared from salt-washed Golgi membranes (11). The high speed supernatant obtained from the rat liver homogenate was used as a source of cytosolic α-D-mannosidase (17). Serotonin-Sepharose 4B (18) was prepared as described (15); agarose-*Ricinus communis* Agglutinin-120 was from Pharmacia LKB Biotechnology Inc.; concanavalin A-Sepharose 4B, wheat germ lectin-Sepharose 6MB, and aprotirin were from Sigma; and endoglycosidase H was from Miles Laboratory Inc. All other

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chemicals used were obtained commercially and were of the highest purity available.

Production of Antiserum—Antiserum against rat liver Golgi mannosidase IA was obtained from a rabbit immunized against homogeneous preparations of this enzyme. Prior to immunization, a male New Zealand White rabbit (1 kg) was bled via an ear vein, and the blood (10 ml) was used for the preparation of preimmune serum. Purified mannosidase IA (~50 μg of enzyme protein) in 1 ml of 20 mM potassium phosphate buffer, pH 7.2, containing 0.2 M NaCl and 0.5% Triton X-100 was emulsified with an equal volume of Freund's complete adjuvant. The rabbit was injected intramuscularly in the thigh and subcutaneously in the back. Immunization was repeated every week for 4 more weeks. Eight days after the last injection, the animal was bled as described above. The blood, allowed to clot at room temperature, was centrifuged at 1600 × *g* for 30 min to obtain serum. The γ-globulin (IgG fraction) was purified from the preimmune serum and antiserum as described (19).

Immunoprecipitation Studies—Washed IgGSorb (100 μl) was incubated at room temperature for 60 min with preimmune IgG fraction or mannosidase IA antibody. The mixture was centrifuged in a MicroCentaur (Accurate Chemical & Scientific Corp.) for 2 min. The pellet was mixed with the appropriate rat liver mannosidase, and the mixture was kept at room temperature for 60 min with occasional mixing. The supernatant solution obtained following centrifugation was assayed for enzyme activity.

Gel Electrophoresis—Polyacrylamide gel electrophoresis was carried out by the method of Davis (20) as described (21). Triton X-100 (0.1, v/v) was present in the sample, gels, and electrophoretic buffer (21). After electrophoresis, the gels were stained for protein with Coomassie Blue (21) or for carbohydrate (22).

SDS[1]-polyacrylamide gel electrophoresis was performed under reducing conditions according to the method of Laemmli (23) as described (24). Gels were stained for protein with 0.5% Coomassie Blue in 20% methanol, 10% acetic acid and destained in 10% methanol, 7% acetic acid.

Molecular Weight Determinations—The oligomeric molecular weight of mannosidase IA was determined by a Ferguson plot of data obtained by polyacrylamide gel electrophoresis according to the method of Hedrick and Smith (25) using the Davis system (20). The subunit molecular weight of the denatured enzyme was determined by the method of Laemmli (23).

Enzyme Assays—Unless otherwise indicated, rat liver lysosomal α-D-mannosidase, Golgi mannosidase II, and cytosolic α-D-mannosidase were assayed in 100 mM sodium acetate buffer at pH 4.4, 5.5, and 6.0, respectively. For these three enzymes, the reaction mixture contained buffer, 4 mM *p*-nitrophenyl-α-D-mannoside, and enzyme in a total volume of 0.5 ml. The incubations were carried out at 37 °C for 15–60 min. The reaction was stopped by the addition of 1.0 ml of an alkaline buffer (0.133 M glycine, 0.067 M NaCl, and 0.083 M Na_2CO_3 adjusted to pH 10.7 (26)). The absorbance at 400 nm was measured to determine the amount of *p*-nitrophenol released. Mannosidases IA and IB were assayed in 50 mM sodium acetate buffer, pH 6.0. The reaction mixture contained buffer, [^{3}H]mannose-labeled oligosaccharide (2,500–10,000 cpm), and enzyme in a total volume of 0.05 ml. Incubation times (at 37 °C) were 10 min to 3 h as indicated in each experiment. The reaction was stopped by heating the sample at 100 °C for 5–7 min. The released [^{3}H]mannose was separated from the oligosaccharide by gel filtration on a column of Bio-Gel P-2 (11) and quantitated by measuring the radioactivity. One unit of the enzyme is the amount which catalyzed the release of 1,000 cpm of [^{3}H]mannose/h.

Oligosaccharides were separated by high resolution Bio-Gel P-4 column chromatography (15, 17). The isolated labeled oligosaccharides were first reduced (27) and subjected to acetolysis (28). The acetolysis products were separated on a Bio-Gel P-4 column (15) and characterized by size.

Total hexose was assayed by the phenol-sulfuric acid method (29) scaled down 5-fold, with mannose as standard. Protein was determined by the fluorometric method of Anderson and Desnick (30) using bovine serum albumin as standard.

Buffers—The following buffers were used in the enzyme purification procedure: Buffer A, pH 7.2 (10 mM potassium phosphate, 0.3% Triton X-100); Buffer B, pH 7.2 (10 mM potassium phosphate, 0.1% Triton X-100); Buffer C, pH 5.8 (10 mM potassium phosphate, 0.1% Triton X-100); and Buffer D, pH 8.0 (10 mM potassium phosphate, 0.1% Triton X-100).

Unless otherwise indicated, Bio-Gel P-2 and P-4 columns were run at room temperature, whereas the enzyme purification steps were carried out at 0–4 °C.

[1] The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

RESULTS

Extraction and Purification of Golgi Mannosidase IA—Salt-washed Golgi membranes obtained from the livers of 16 rats (10) were extracted by suspending the membranes in Buffer A (5–6 mg protein/ml of buffer), followed by homogenization in a glass homogenizer with a Teflon pestle rotating at 1,200 rpm (six up and six down strokes). The suspension was centrifuged at 50,000 rpm (165,000 × *g*) for 30 min. The supernatant was removed by aspiration, and the pellet was resuspended in 5–6 ml of the same buffer and homogenized and centrifuged again as described above. This process was repeated two more times.

The combined supernatant solution (~25 ml), which contained nearly 80% of the protein and over 85% of α1,2-mannose cleaving activity with [^{3}H]$Man_8GlcNAc$ as substrate, was used for the enzyme purification. The enzyme present in the supernatant solution was precipitated at 60% saturated $(NH_4)_2SO_4$. The mixture was kept on ice for 15–20 min and then centrifuged at 50,000 rpm for 30 min. The precipitated enzyme, containing essentially all of the α1,2-mannosidase activity, was dissolved in a small volume of Buffer B (4–5 ml).

The slightly turbid enzyme solution was applied to a Sephacryl S-300 column (1.5 × 83 cm) equilibrated with Buffer B. After 10 fractions were collected (Fig. 1), 6 ml of 18% $(NH_4)_2SO_4$ in Buffer B was applied to the column, followed by elution with Buffer B. The enzymatic activity was eluted in three components. Nearly 50% of the enzyme applied to

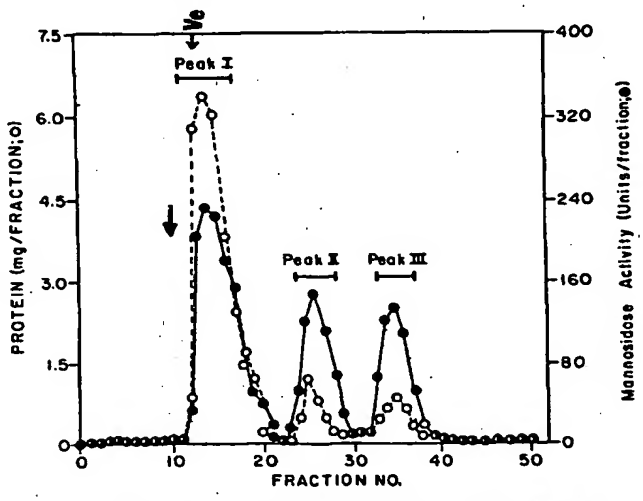

FIG. 1. **Chromatographic separation of mannosidase IA from mannosidase IB on Sephacryl S-300.** The α1,2-specific mannosidase activities present in the Triton X-100 extract of rat liver Golgi membranes were precipitated with 60% $(NH_4)_2SO_4$. The residue was suspended in a small volume of Buffer B (~5 ml), and the suspension was applied to a Sephacryl S-300 column (1.5 × 83 cm) equilibrated with this buffer. Fractions (4.8 ml) were collected at a flow rate of 8 ml/h. After 10 fractions were collected, 6 ml of 18% $(NH_4)_2SO_4$ in Buffer B was applied to the column (indicated by the *arrow*), followed by elution with Buffer B. Each fraction was analyzed for α-D-mannosidase activity using [^{3}H]$Man_8GlcNAc$ as substrate (●) and for protein (○) as described under "Experimental Procedures." The V_e indicated for blue dextran 2000 shows the column void volume.

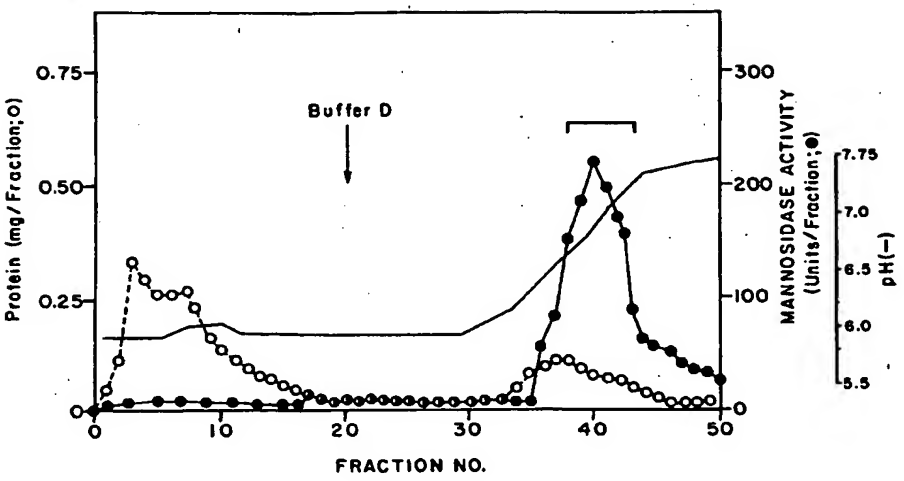

FIG. 2. **Chromatography of mannosidase IA on cellulose phosphate.** Enzymatically active fractions (Peaks II and III) from the Sephacryl S-300 column (Fig. 1) were pooled, and the pH was adjusted to 5.8. The sample was dialyzed for 4 h against 50 volumes of Buffer C with two changes. The dialyzed enzyme was applied to a cellulose phosphate column (1 × 14 cm) equilibrated with Buffer C. The column was washed with 50 ml of Buffer C, followed by elution with Buffer D. Fractions (4.0 ml) were collected at a flow rate of 10 ml/h. Each fraction was checked for pH using a digital ion analyzer (Orion Research Inc.). Aliquots from each fraction were also analyzed for enzymatic activity (●) and protein content (○) as described in the legend to Fig. 1. Fractions 38–43 were pooled and used in the next step of purification.

the column was present in the void volume fractions (Fig. 1, *Peak I*). The enzymatic activity present in Peak I fractions was found to bind quantitatively to a cellulose phosphate column at pH 7.2 and could be eluted with 0.15 M NaCl, a result suggesting that this activity was due to mannosidase IB (11). However, the activity present in the later fractions from the Sephacryl S-300 column (Fig. 1, *Peaks II* and *III*) did not bind to a cellulose phosphate column at pH 7.2, suggesting that this activity was due to mannosidase IA (11).

The elution of mannosidase IA as two peaks deserves comment. We were faced with the fact that (i) mannosidase IA apparently adsorbs to Sephacryl S-300, and (ii) residual ammonium sulfate in the enzyme to be applied to the column could not be removed by dialysis from the concentrated solution of crude enzyme without appreciable loss of enzymatic activity. In the procedure ultimately developed (Fig. 1), the residual ammonium sulfate is responsible for the elution of the mannosidase IA in Peak II of Fig. 1. The ammonium sulfate solution added to the column (Fig. 1, *arrow*) causes the elution of all of the remaining mannosidase IA (Fig. 1, *Peak III*). The activities in the pools comprising Peaks II and III showed identical behavior on a cellulose phosphate column, and the combined pools (Peaks II and III) yielded homogeneous mannosidase IA, as described later, in 11 highly reproducible experiments.

Fractions containing mannosidase IA (Fig. 1, *Peaks II* and *III*) were pooled, adjusted to pH 5.8, and dialyzed against 50 volumes of Buffer C for 4 h with two changes of the buffer. The dialyzed enzyme was applied to a cellulose phosphate column (1 × 14 cm) equilibrated with Buffer C. The column was washed with 50 ml of the buffer, followed by elution with 150 ml of Buffer D. Fractions were collected and checked for pH. Aliquots from each fraction were assayed for protein and enzymatic activity. Most of the protein was found in the void volume fractions which showed no mannosidase activity (Fig. 2). The enzyme began to appear in the eluate at pH 6.25. The peak of elution occurred at pH 6.8 (Fig. 2). Pooled fractions 38–43 showed a specific activity of over 2500, which corresponds to nearly 70-fold purification over the Golgi membranes (Table I).

The pooled fractions from the cellulose phosphate column were applied to a DE 52 column (1 × 14 cm) equilibrated with Buffer B. After the column was washed with 50 ml of this buffer, it was eluted with 200 ml of a linear NaCl gradient (0–0.5 M) in this buffer. Fractions (4 ml) were collected at a flow rate of 10 ml/h. Nearly 30% of the original enzyme activity was present in fractions 7–10.

TABLE I

Purification of mannosidase IA from rat liver Golgi-rich fraction

Purification step	Protein		[^{3}H]Man$_8$-mannosidase[a]		
	Amount	Yield	Activity	Specific activity	Yield
	mg	%	*units*	*units/mg*	%
Golgi-rich fraction	64	100	2440	38	100
Triton X-100 extract	50	78	2160	43	88
$(NH_4)_2SO_4$ precipitation	42	66	2080	50	85
Sephacryl S-300[b]	5.2	8.1	1070	206	44
Cellulose phosphate	0.36	0.56	930	2580	38
DEAE-cellulose (DE52)	0.12	0.19	760	6330	31
Hydroxylapatite	0.065	0.11	460	7080	19

[a] The enzyme was assayed using [^{3}H]Man$_8$GlcNAc as substrate as described under "Experimental Procedures."

[b] The enzymatically active fractions in Peaks II and III (Fig. 1) were pooled, dialyzed, and used for further purification of mannosidase IA.

The enzymatically active fractions were pooled and applied to a hydroxylapatite column (1 × 5 cm) equilibrated with Buffer B. The column was washed with 50 ml of the above buffer, followed by elution with 100 ml of a linear potassium phosphate gradient (10–500 mM, pH 7.2, containing 0.1% Triton X-100). One-ml fractions were collected and assayed for mannosidase activity. Nearly 20% of the original enzymatic activity eluted at 35–60 mM phosphate (fractions 5–7). These fractions were pooled and concentrated to 1 ml using a Centricon™ microconcentrator (Amicon Corp.).

Results obtained from a typical experiment are shown in Table I. The enzymatic activity toward [^{3}H]Man$_8$GlcNAc was purified over 160-fold from the Golgi membrane extract with nearly 20% recovery. Since the extract contained both mannosidase IA and IB activities in approximately equal amounts (Ref. 11; see also Fig. 1), the actual purification of mannosidase IA was approximately 300-fold, and the recovery was about 40%. The purified, concentrated mannosidase IA showed no *p*-nitrophenylmannosidase activity even when the incubations (37 °C) were carried out for 4 h.

We previously described the separation and preliminary characterization of mannosidases IA and IB from rat liver Golgi membranes (11). The two enzymes were quite similar in their substrate specificities and response to certain inhibitors (11). However, their behavior on a cellulose phosphate

column was quite different, and only mannosidase IB showed some activity toward *p*-nitrophenylmannoside (11). This activity has, as expected, a pH optimum of 6.0.[2] In this study, we show that the two enzymes can also be separated on a column of Sephacryl S-300 (Fig. 1). That the enzyme activity present in the column void volume fractions is indeed mannosidase IB was shown by the following experiments. (*a*) Even after two cycles of precipitation with $(NH_4)_2SO_4$ and chromatography of Peak I fractions on the Sephacryl S-300 column, over 80% of the activity was present in the void volume fractions. (*b*) This enzyme bound to a cellulose phosphate column at pH 7.2 and was eluted with 0.15 M NaCl. (*c*) The void volume fractions showed some activity toward *p*-nitrophenyl-α-D-mannoside. The mannosidase activity purified to homogeneity was characterized as mannosidase IA on the basis of (*a*) its inability to hydrolyze *p*-nitrophenyl-α-D-mannoside and (*b*) its behavior on the cellulose phosphate column (11).

Several lines of evidence reported in our previous publication (11) suggested that mannosidases IA and IB are not proteolytic products. We have now performed an additional experiment bearing on this question by including a mixture of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, leupeptin (1 μg/ml), aprotinin (0.1 trypsin inhibitor unit/ml), and pepstatin (2 μg/ml). Rat liver was homogenized in sucrose solution containing the inhibitors. The salt-washed Golgi membranes were prepared from this homogenate by our published procedure (10), except that all solutions used contained the inhibitors. Furthermore, the protease inhibitors were also present in the extraction and purification buffers. When the $(NH_4)_2SO_4$-precipitated enzyme was resolved on a Sephacryl S-300 column as described for Fig. 1, the α1,2-mannosidase activities eluted in three peaks. The ratio of the enzyme activities present in each of the three peaks was quite similar to the results shown in Fig. 1. These results are consistent with the assumption that the two mannosidases are not generated by proteolytic cleavage. When the enzymatically active fractions from Peaks II and III were pooled and the purification scheme was carried out through the hydroxylapatite column, the purified enzyme again appeared on an SDS-PAGE gel as a single peptide of M_r 57,000 (see below).

Evidence for Purity of Mannosidase IA—The purity of mannosidase IA was examined by analytical gel electrophoresis in the Davis system (20) as described under "Experimental Procedures." When 5–7 μg of the enzyme protein was electrophoresed and stained for protein, the enzyme showed a single band (Fig. 3). To determine whether the enzymatic activity was present at the position of the protein band, two gels were run side-by-side; one was stained for protein, and the other was cut into 20 equal slices. Each slice was suspended (with a homogenizer) in 0.1 ml of 0.2% Triton X-100, 0.25 M NaCl. The suspension of the slice corresponding to the protein band contained 35–40% of the original enzymatic activity. No other slice yielded any enzymatic activity, except for a small amount of activity at the origin. When the suspension of the gel slice containing mannosidase IA was centrifuged, the supernatant solution was found to be devoid of activity. Electrophoresis of the native enzyme in gels of varying polyacrylamide concentration yielded gels showing only one component and, by Ferguson plot (25), gave an M_r of 230,000 ± 8,000 (Fig. 4).

When 1.4–5.6 μg of enzyme protein was resolved on SDS-PAGE and stained for protein, the denatured enzyme showed a single protein band of M_r 57,000 (Fig. 5). However, electrophoresis of less than 1 μg of enzyme protein gave a faint doublet of M_r 57,000 and 58,000 (data not shown). The results of electrophoresis of the native and denatured enzyme indicate that mannosidase IA is a tetramer containing four very similar subunits.

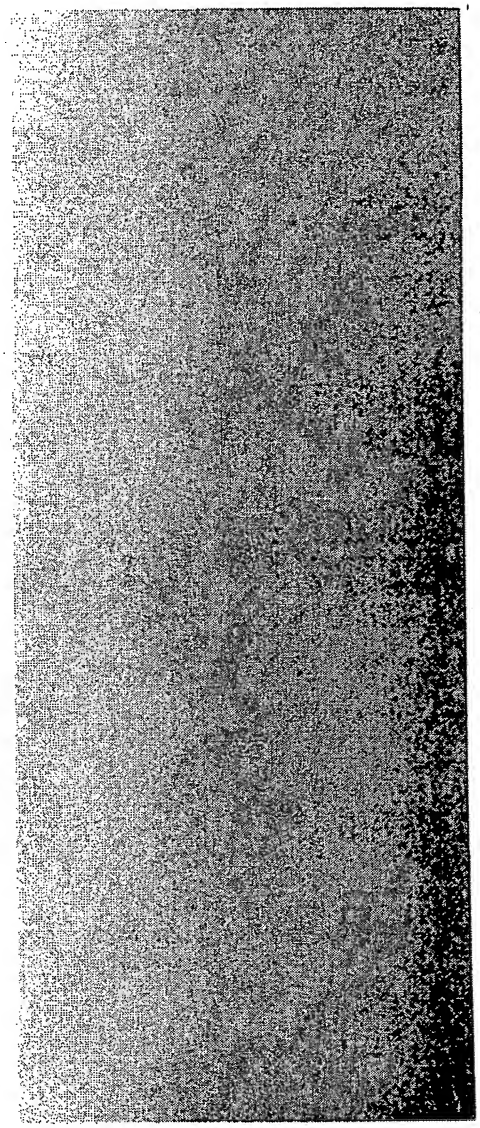

FIG. 3. **Analytical gel electrophoresis of mannosidase IA.** Purified rat liver Golgi mannosidase IA (7 μg of enzyme protein) was mixed in 10% sucrose and applied to 7% polyacrylamide gel. The gel was subjected to electrophoresis in the cold room by applying a constant current of 2 mA/gel. After electrophoresis, the gel was stained for protein with Coomassie Blue (21). Electrophoresis was from top to bottom toward the anode (20).

Glycoprotein Nature of Mannosidase IA—When 40–50 μg of the enzyme protein was resolved in the Davis electrophoresis system (20) and stained by periodic acid-Schiff's base, a faint periodic acid-Schiff's base-positive band was seen. However, extensive washing of the gel resulted in disappearance of the band. When the native enzyme was applied to a concanavalin A-Sepharose 4B column (31, 32) and washed immediately, the enzyme did not bind to this affinity column. However, over 70% of the enzyme did bind to the column if it was left in contact with the lectin column overnight. The

[2] D. R. P. Tulsiani and O. Touster, unpublished data.

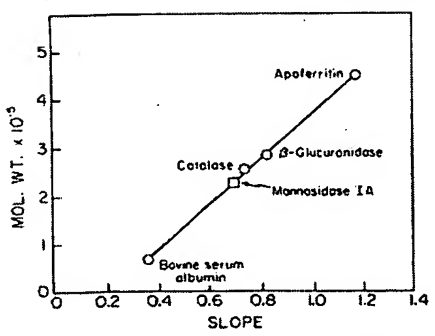

FIG. 4. **Determination of oligomeric molecular weight of Golgi mannosidase IA by polyacrylamide gel electrophoresis.** Five μg of the purified mannosidase IA and 10 μg each of apoferritin (M_r 445,000), preputial gland β-glucuronidase (M_r 280,000), catalase (M_r 250,000), and bovine serum albumin (M_r 66,000) were electrophoresed on separating gels of 4, 5, 6, and 7% acrylamide (20). The gels were stained for proteins with Coomassie Blue (21). The mobility of each protein was expressed as a ratio relative to the dye front (25). The log of the mobility of each protein was plotted against the gel concentration, and the resulting slope was plotted against the known molecular weight of the protein.

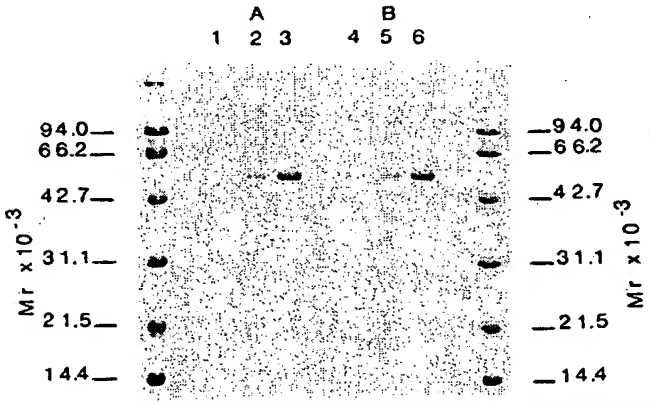

FIG. 5. **Electrophoretic behavior of purified mannosidase IA on SDS-polyacrylamide gel.** SDS-PAGE electrophoresis (Laemmli system (23)) was carried out on two different preparations (*A* and *B*) of the purified mannosidase IA (15% gel). Standard marker proteins are to *right* and *left*. The other lanes are different amounts of enzyme protein of preparations *A* and *B*. *Lanes 1* and *4*, 1.4 μg of protein; *lanes 2* and *5*, 2.8 μg of protein; *lanes 3* and *6*, 5.6 μg of protein. A single band of M_r 57,000 was observed. The gel was stained for proteins as described under "Experimental Procedures."

binding appears to be due to the presence of glucose and/or mannose in the enzyme because nearly 50% of the enzymatic activity could be eluted with 1 M α-methylmannoside. In addition, the treatment of the native enzyme with endoglycosidase H as described (15), an enzyme that removes high mannose and hybrid oligosaccharides (15), eliminated its binding to the lectin. However, this treatment did not produce a detectable change in the size of the enzyme subunit. In one experiment, two preparations of purified enzyme were pooled, dried in a Bio-Drier, and analyzed for total hexose as described (29). The enzyme contained only 0.88% hexose.

That the enzyme does not contain terminal neuraminic acid or galactose or peripheral *N*-acetylglucosamine, sugars commonly found in complex glycoproteins, was shown with sugar-specific lectin columns. The enzyme did not bind to serotonin-Sepharose 4B (specific for neuraminic acid (15, 18)), ricin (galactose), or wheat germ lectin (*N*-acetylglucosamine), although labeled glycopeptides of known structure showed the predicted specificity for these affinity columns (data not shown). On the basis of these results, we suggest that mannosidase IA probably contains one to two chains of high mannose oligosaccharides.

Stability of Mannosidase IA—Purified concentrated mannosidase IA was very stable when stored at 0–4 °C in solutions containing 10 mM potassium phosphate buffer, pH 7.2, and 0.5% Triton X-100. Under these conditions, the enzyme could be stored for 4–6 months without appreciable loss of activity.

Kinetics and Substrate Specificity—Purified mannosidase IA showed maximum activity at pH 6.0, in agreement with the value reported for less purified enzyme (11). Under standard assay conditions, the rates of hydrolysis of substrates ranging from $Man_9GlcNAc$ to $Man_6GlcNAc$ were directly proportional to the enzyme concentration (20–80 ng of enzyme protein). With 70 ng of the enzyme, the reaction was linear for 10–40 min depending on the substrate used (Fig. 6). The amount of free [^{3}H]mannose released from each substrate at any specific time depended on the size of the oligosaccharide used (Fig. 6*A*), with $Man_9GlcNAc$ yielding the most [^{3}H]mannose and $Man_6GlcNAc$ the least. However, when [^{3}H]mannose release is plotted as a percentage of available mannosyl residues (*i.e.* α1,2-linked residues) (Fig. 6*B*), it is evident

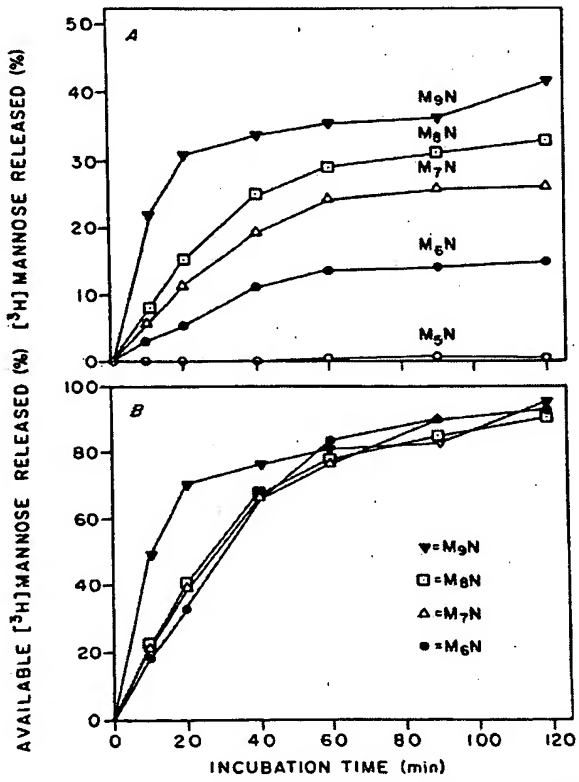

FIG. 6. **Time course of hydrolysis of [^{3}H]mannose-labeled oligosaccharides by purified mannosidase IA.** Approximately 2500 cpm of each of the five oligosaccharides was incubated at 37 °C with homogeneous mannosidase IA (70 ng of enzyme protein) for various time periods. The released [^{3}H]mannose was quantitated after separation from the labeled oligosaccharides by gel filtration on a column of Bio-Gel P-2 as described under "Experimental Procedures." In *A*, the release of [^{3}H]mannose is expressed as percentage of total mannose in the substrate. In *B*, it is expressed as percentage of available (*i.e.* α1,2-linked) mannosyl residues. For example, M_9N, $Man_9GlcNAc$.

that the Man_9 derivative clearly contains a more susceptible α1,2-mannosyl linkage than is present in the other three substrates. This inference is in accord with evidence given later in this paper on the sequence of mannose removal. $Man_5GlcNAc$, which contains no α1,2-linked mannosyl residues, showed only low activity as a substrate (Fig. 6*A*).

Effectors—Cu^{2+}, Co^{2+}, Tris, EDTA, and *p*-chloromercuriphenylsulfonic acid were potent inhibitors of purified mannosidase IA, as described earlier for the partially purified enzyme (11). Ca^{2+} and Mg^{2+} at 1 and 10 mM concentrations did not affect enzyme activity. The enzyme is inhibited by the mannose analogue 1-deoxymannojirimycin, with 1 μM concentration inhibiting 50%. However, a small fraction of activity (nearly 5%) remained even at a 1-deoxymannojirimycin concentration of 2 mM (Fig. 7). Whether or not this residual activity represents a different enzyme species is unknown. In similar studies, we have found that Golgi mannosidase IB is also inhibited by 1-deoxymannojirimycin (data not shown), which has little or no effect on liver cytosolic and lysosomal α-D-mannosidases or on Golgi mannosidase II (33).

That the 1-deoxymannojirimycin inhibition of mannosidase IA is largely reversible is shown in Table II. In this experiment, the enzyme was mixed with various concentrations of 1-deoxymannojirimycin and assayed before and after dialysis. It is obvious from the results in Table II that over 70% of the enzyme activity was recovered after 1-deoxymannojirimycin was removed by dialysis. These results obtained with the purified enzyme are consistent with cell culture experiments, which suggested that the 1-deoxymannojirimycin inhibition of mannosidase IA is reversible (34).

Requirement of Detergent for Enzymatic Activity—The purified, concentrated enzyme contained 0.5% Triton X-100 as assayed by the method of Holloway (35). The enzyme remained completely soluble and fully active when centrifuged at 165,000 × *g* for 60 min. Removal of the detergent with Bio-Beads resulted in loss of enzymatic activity. As shown in Fig. 8, the enzyme is nearly 100% active at a detergent concentration of 0.12% and higher. However, nearly 60% of the activity was lost when the detergent concentration reached 0.09%, and no activity remained when the detergent concentration was reduced to 0.07% (Fig. 8). That the loss of enzymatic activity was not due to the precipitation of enzyme was shown by comparing the protein concentration in the 165,000 × *g* supernatant before and after removal of the detergent. Over 80% of the original protein was still in the supernatant after detergent removal. It is unclear why the enzymatic activity was lost when an appreciable concentration of detergent was still present.

Because an α1,2-specific mannosidase purified from rabbit liver microsomes was reported to be markedly stimulated by Triton X-100 and phosphatidylethanolamine (36), we attempted to reactivate mannosidase IA. Only about 30% of the original enzymatic activity was recovered when the detergent concentration was increased to 0.5% and the phosphatidylethanolamine concentration to 100 μg/0.05 ml. These studies suggest that Golgi mannosidase IA is perhaps different from the rabbit liver enzyme. However, this study and that of previous workers (36) should be compared with caution for

TABLE II

Study of reversibility of 1-deoxymannojirimycin inhibition of mannosidase IA

Purified mannosidase IA (~40 units) in 10 mM potassium phosphate, pH 7.2, containing 0.2% Triton X-100 was mixed with different concentrations of 2-deoxymannojirimycin and kept on ice for 15 min. Aliquots from each sample were analyzed for enzyme activity before and after dialysis (4 h at 0–4 °C) against 1000 volumes of 10 mM potassium phosphate buffer, pH 7.2, containing 0.2% Triton X-100.

Preincubation (additions)	Enzyme activity			
	Before dialysis		After dialysis	
	Units	%	Units	%
None	42	100	37	100
1-Deoxymannojirimycin				
0.1 mM	9	21	32	86
0.5 mM	5	12	28	76
1.0 mM	4	10	30	81
2.0 mM	2	5	27	73

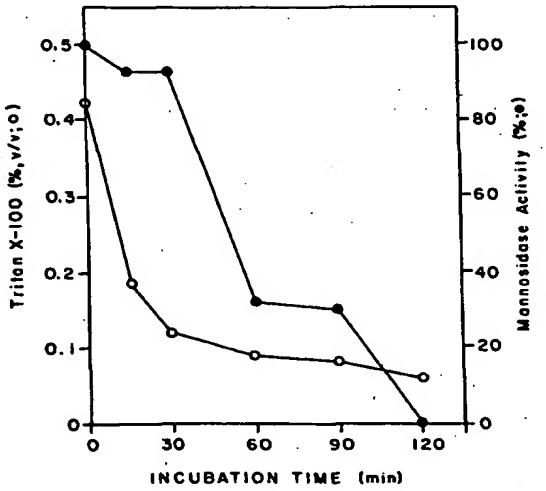

FIG. 8. **Effect of removal of Triton X-100 on enzymatic activity of mannosidase IA.** Homogeneous mannosidase IA (5 μg of enzyme protein) in 0.5 ml of 10 mM potassium phosphate buffer, pH 7.2, containing 0.5% Triton X-100 was mixed with 0.6 g of moist Bio-Beads SM-2 (10), and the mixture was kept on ice with occasional mixing. At various time intervals, aliquots were withdrawn and assayed for detergent concentration (35) and enzymatic activity using [^{3}H]$Man_9GlcNAc$ as substrate (11).

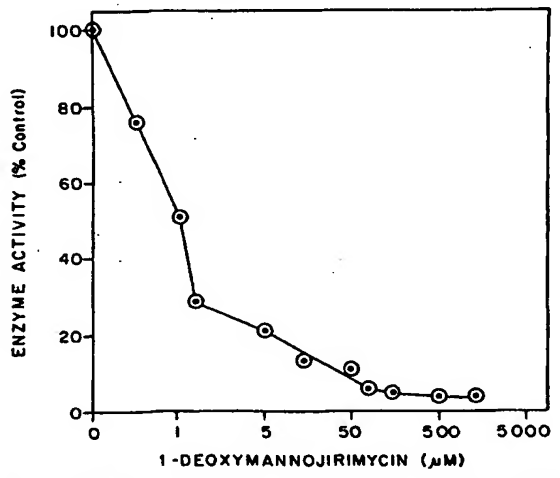

FIG. 7. **Effect of 1-deoxymannojirimycin on purified mannosidase IA.** Homogeneous Golgi mannosidase IA (50 ng of enzyme protein) was first preincubated (15 min at 0–4 °C) with varying concentrations of 1-deoxymannojirimycin at pH 6.0 before addition of [^{3}H]$Man_9GlcNAc$. The reaction mixtures were incubated at 37 °C for 15 min. The released [^{3}H]mannose was quantitated after separation from labeled oligosaccharides as described under "Experimental Procedures."

the following reasons: (i) the unknown effect of contaminating macromolecules present in the partially purified rabbit liver mannosidase (36), (ii) the quite different substrates used in the two reports, and (iii) unknown effects of Bio-Beads used in our studies. The procedures used for depleting the detergent concentration with the rabbit enzyme are not apparent. A more meaningful comparison of the two enzymes will require studies done under identical conditions.

Immunoprecipitation Studies—Polyclonal antibody to rat liver Golgi mannosidase IA was used to study immunological relationships among various rat liver mannosidases. As shown in Fig. 9, the liver cytosolic and lysosomal α-D-mannosidases as well as Golgi mannosidase II showed no cross-reactivity with the antibody. However, both mannosidases IA and IB reacted with the antibody.

Order in Which Mannose Residues Are Released from $Man_9GlcNAc$—To investigate the order in which the 4 α1,2-mannosyl residues are removed from $Man_9GlcNAc$, we studied the time course of the hydrolysis of [^{3}H]$Man_9GlcNAc$ by purified mannosidase IA (Fig. 10, *upper*). At several time points, the reaction intermediates in the incubation mixtures were isolated by high resolution gel filtration on Bio-Gel P-4 (Fig. 10, *lower*), yielding oligosaccharides ranging from $Man_9GlcNAc$ (*Panel A*, 0 h) to $Man_5GlcNAc$ (*Panel E*, 2 h). As previously reported (11), a small amount of $Man_4GlcNAc$ is also ultimately formed (*Panel E*). Each of the major oligosaccharides obtained in this fractionation was reduced and subjected to acetolysis, a reaction that rather selectively cleaves α1,6-linkages. The acetolysis products were then fractionated on a Bio-Gel P-4 column (Fig. 11). The composition of each acetolysis product was deduced from its elution position. The products obtained from $Man_9GlcNAc$, shown in Fig. 11*A*, consisted of an oligosaccharide which migrated at the position of $Man_4GlcNAc$ and oligosaccharides that migrated as Man_3 (equivalent to ManGlcNAc) and Man_2. These are the expected products from $Man_9GlcNAc$ as shown in the structure in Fig. 11*A*. The $Man_8GlcNAc$ reaction intermediate gave acetolysis products ($Man_4GlcNAc$ and Man_2) consistent with the structure shown (Fig. 11*B*). In Fig. 11*C*, the larger cleavage products eluted at the positions of $Man_4GlcNAc$ and $Man_3GlcNAc$, suggesting that the processing intermediate

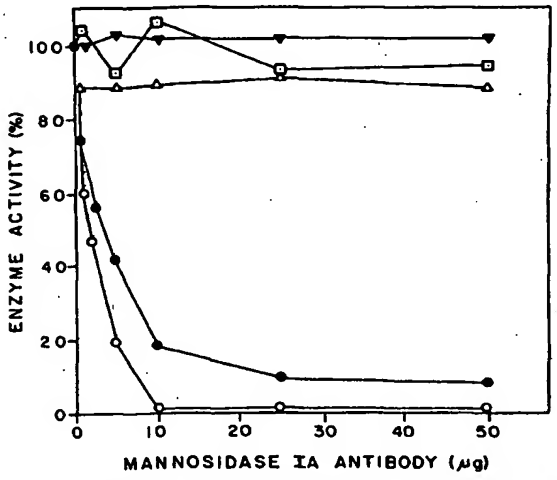

FIG. 9. **Immunoprecipitation of rat liver mannosidases using anti-mannosidase IA antibody (IgG).** Various mannosidases prepared from rat liver were added to 100 μl of prewashed IgGSorb to which the indicated amounts of the anti-mannosidase IA antibody had been preadsorbed. This suspension was kept at room temperature for 60 min with occasional mixing. The mixture was centrifuged in a microcentrifuge for 2 min. The enzymatic activity remaining in the supernatant was determined as described under "Experimental Procedures" and plotted against the concentration of antibody. △, Golgi mannosidase II; □, cytosolic α-D-mannosidase; ▼, lysosomal α-D-mannosidase; ○, Golgi mannosidase IA; ●, Golgi mannosidase IB. Mannosidases IA and IB were quantitatively recovered in the supernatant solution when IgG from preimmune serum was used.

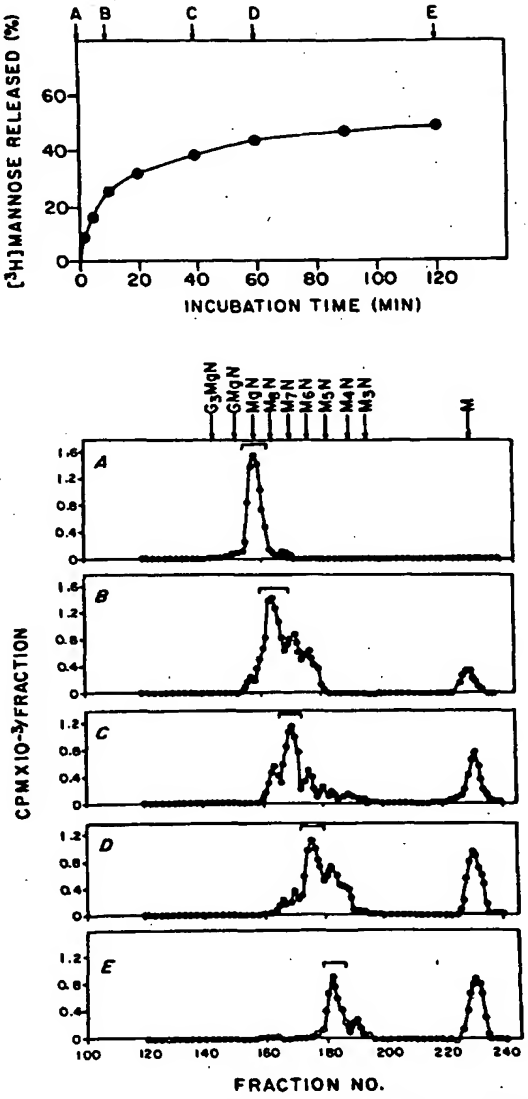

FIG. 10. **Time course of hydrolysis of [^{3}H]$Man_9GlcNAc$ by purified mannosidase IA.** Approximately 10,000 cpm of the oligosaccharide was incubated in sodium acetate buffer, pH 6.0, at 37 °C with homogeneous mannosidase IA (70 ng of enzyme protein). At the indicated time periods (*upper*), aliquots were withdrawn, and the released [^{3}H]mannose was quantitated after separation from ^{3}H-labeled oligosaccharides on a column of Bio-Gel P-2 (11). In a separate set of experiments, at the indicated time points (*upper, points A–E*), the reaction was stopped by heat treatment (5 min at 100 °C), and the mixture was resolved by high resolution chromatography on a column of Bio-Gel P-4 (15). The oligosaccharides in *panels A–E* (*lower*) were pooled (shown by the *horizontal bar*) and Bio-Dried. The residual oligosaccharides were reduced and subjected to acetolysis as described under "Experimental Procedures." G_3M_9N, for example, $Glc_3Man_9GlcNAc$.

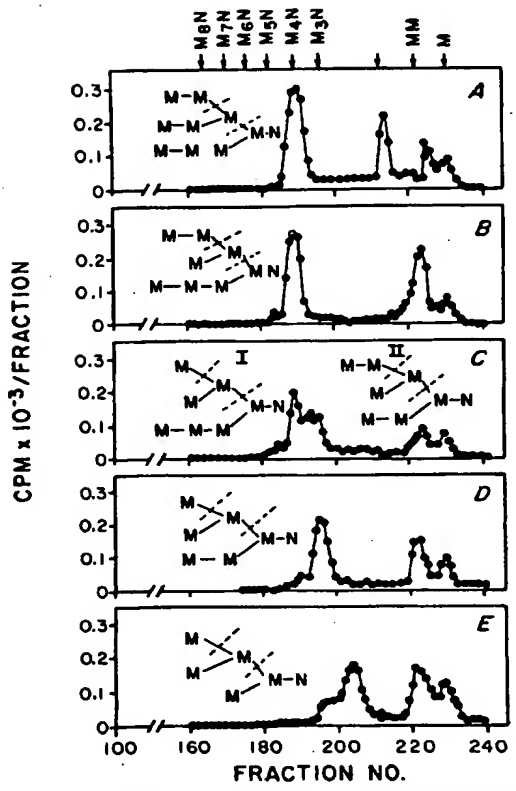

FIG. 11. **Fractionation of [^{3}H]mannose-labeled products following acetolysis of oligosaccharide pool in *panels A–E* of Fig. 10 (*lower*).** Following reduction and acetolysis, the reaction mixtures were resolved on Bio-Gel P-4 column (15). The assigned structure of each oligosaccharide alcohol, shown in each panel, is based on the analysis of acetolysis products and on the size of the original oligosaccharide (Fig. 10). The *dashed lines* in the structures are expected sites of cleavage. Over 90% of the radioactivity present in each of the acetolysis mixtures was recovered in the fractions from each Bio-Gel P-4 column (*A–E*).

$Man_7GlcNAc$ is a mixture of two isomers. The fact that there was more of $Man_4GlcNAc$, than $Man_3GlcNAc$ (Fig. 11*C*) indicates that isomer I is the major product. As expected from the structure shown for $Man_6GlcNAc$, this intermediate yielded $Man_3GlcNAc$, Man_2, and mannose (Fig. 11*D*). $Man_5GlcNAc$ yielded an oligosaccharide peak which appeared between $Man_3GlcNAc$ and ManGlcNAc and is presumably $Man_2GlcNAc$ (Fig. 11*E*).

DISCUSSION

Rat liver Golgi mannosidase I was partially purified and characterized by Tabas and Kornfeld (9). Subsequently, we showed that the enzyme could be fractionated into two distinct activities (mannosidases IA and IB) on a column of cellulose phosphate (11). The enzymatic activity present in the column void volume was unable to cleave *p*-nitrophenyl-α-D-mannoside and was designated mannosidase IA (11). However, the activity which was eluted from the cellulose phosphate column with a salt gradient showed some activity toward this synthetic substrate and was designated mannosidase IB (11). In addition, the two enzymes showed a significant difference in (*a*) thermolability and (*b*) their activity toward $Man_8GlcNAc$ (11). In this study, we separated the two activities on a column of Sephacryl S-300. The retarded/adsorbed activity (mannosidase IA) has been purified to homogeneity.

Homogeneous mannosidase IA has a subunit molecular weight of 57,000 and 58,000 and an oligomeric molecular weight of approximately 230,000, suggesting that the enzyme is a tetramer consisting of four very similar subunits. Based on its binding to concanavalin A and its sensitivity to endoglycosidase H, the enzyme appears to be a glycoprotein. With a hexose content of at most 1%, the enzyme can contain only one or two oligosaccharide chains. Since the enzyme appeared as a doublet in SDS-PAGE, it is possible that all subunits are not similarly glycosylated. The enzyme showed no evidence for the presence of the terminal sugars (*i.e.* sialic acid, galactose) and peripheral *N*-acetylglucosamine characteristic of complex glycoproteins. This result is in agreement with the suggested localization of the enzyme in the cis region of the Golgi complex (37–39).

Substrate specificity studies described here are in agreement with our earlier results with the partially purified enzyme (11). The homogeneous enzyme shows highest activity toward $Man_9GlcNAc$ and decreasing activity through the series of processing intermediates, $Man_8GlcNAc$ to $Man_5GlcNAc$, when the results are plotted as percentage of total mannose present (Fig. 6*A*). However, as shown in Fig. 6*B*, 1 residue in the Man_8 derivative is especially susceptible to cleavage. Analysis of the various products isolated during the degradation of $Man_9GlcNAc$ by the enzyme indicated that the terminal α1,2-linked mannose residue on the middle antenna is the first sugar to be cleaved, producing a single $Man_8GlcNAc$ isomer. This result, although differing from an earlier report based on the use of a partially purified enzyme (9), is in agreement with several *in vivo* studies on the processing of mammalian glycoproteins (27, 40, 41). The $Man_8GlcNAc$ isomer is then cleaved to form two $Man_7GlcNAc$ isomers (Fig. 11), which are further processed to a single $Man_6GlcNAc$ isomer. The removal of the last α1,2-mannosyl residue linked to the α1,3-mannose in $Man_6GlcNAc$ (Fig. 11) makes accessible the mannosyl residue in $Man_5GlcNAc$ which is *N*-acetylglucosaminylated by GlcNAc-transferase I (42, 43).

Immunoprecipitation studies showed that antibody raised against the homogeneous preparations of mannosidase IA cross-reacted with mannosidase IB. This finding was not very surprising since these two enzymes show similar catalytic properties (11). We have previously shown that β-glucuronidases prepared from rat liver lysosomes and microsomes, in spite of the differences in their charge, carbohydrate content, and amino acid content (22), cross-reacted with the antibody to preputial gland β-glucuronidase (21). It is conceivable that mannosidase IA and IB represent two isoforms of the same molecule, a situation similar to forms A and B of human liver lysosomal α-D-mannosidase. This enzyme has been shown to be composed of two immunologically identical charge isomers (44). Perhaps the most interesting and relevant example of cross-reactivity is the reaction of a novel brain microsomal α-D-mannosidase with antibody to rat liver cytosolic α-D-mannosidase even though the two enzymes show marked differences in their properties, including substrate specificity (45). Mannosidases IA and IB may contain a common subunit, or they may be derived from a common precursor.

The antibody was also used to examine a microsomal membrane extract for possible evidence of additional mannosidase activities. Immunoprecipitation of $Man_8GlcNAc$-cleaving activity over a wide range of antibody concentrations gave results indicating that nearly 90% of the enzyme activity present in the microsomes cross-reacted with the antibody.[2] This finding was supported by the fact that over 90% of the

α1,2-mannosidase activity in the extract was sensitive to 1-deoxymannojirimycin. These experiments clearly indicate that mannosidase IA + IB are by far the predominant enzymes in microsomal membranes possessing α1,2-mannosidase activity. Endoplasmic reticulum mannosidase is not inhibited by 1-deoxymannojirimycin (33).

Several glycoproteins of known endoplasmic reticulum localization have been reported to contain high mannose oligosaccharides, varying in size from $Man_8GlcNAc$ to $Man_6GlcNAc$ (46–50). These findings are consistent with the reported occurrence of one or more mannosidase activities in endoplasmic reticulum (7, 8). A second pathway for the production of such oligosaccharides was recently reported (51), namely, the direct transfer of nonglucosylated oligosaccharide(s) from lipid to protein.

We have no explanation as to why mannosidase IB behaves as a very large molecule, eluting in the void volume fractions from a Sephacryl S-300 column. This property is not artifactual since rechromatography did not change its behavior on this column. Similarly, rechromatography of mannosidase IA did not yield any activity in the void volume fractions. It is possible that mannosidase IB occurs as an aggregate, perhaps bound to lipid and/or polysaccharide.

A processing mannosidase has recently been purified and characterized from calf liver microsomes (52). This enzyme, like rat liver mannosidase IA, is reported to be an α1,2-mannosidase. Both enzymes are very sensitive to the mannose analogue 1-deoxymannojirimycin. In addition, the reported subunit molecular weight of the calf liver enzyme (56,000) is very similar to the mannosidase IA subunit(s) (57,000–58,000). However, the mannosidases from the livers of the two species show different substrate specificity. Rat liver mannosidase readily cleaves all 4 α1,2-linked mannosyl residues from $Man_9GlcNAc$ (Fig. 10), whereas the enzyme from calf liver cleaves only 3 α1,2-linked mannosyl residues (52). In addition, an α1,2-specific mannosidase purified from rabbit liver (36) also appears to be different from mannosidase IA because the former enzyme is reported to be activated by detergent and/or phospholipids (36). As described here, the addition of Triton X-100 and/or phosphatidylethanolamine to mannosidase IA does not appreciably reactivate the enzyme.

There is little basis for speculating on whether mannosidases IA and IB have different physiological roles. The two enzymes are present in approximately equal activities in the Golgi apparatus (11). It is possible that the two enzymes are selectively involved in different processing routes, each enzyme responsible for cleaving particular mannose residues from *N*-linked glycoprotein. Moreover, since the Golgi apparatus is a complex system of cisternae, it is possible that each enzyme has a selective localization in these cisternae. There is in fact evidence from structural studies on IgM suggesting that the high mannose oligosaccharides on asparagines 402 and 563 are processed by different sequences of mannose removal (53). It will therefore be important to obtain highly purified mannosidase IB so that the steps in the processing of $Man_9GlcNAc$ by this enzyme can also be determined.

The availability of antibody to mannosidase IA should be useful in studies involving biosynthesis and regulation of this enzyme. In addition, the antibody should be helpful in studying the membrane topology and translocation of the enzyme by approaches including immunocytochemistry. Results from these studies will further our understanding of the biosynthesis, processing, and transport of *N*-linked glycoproteins in mammalian cells.

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Purification of two isozymes of rat liver microsomal cytochrome P450 with testosterone 7 alpha-hydroxylase activity.

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Cytochrome P450a was purified to electrophoretic homogeneity from liver microsomes from immature male Long-Evans rats treated with Aroclor 1254 Rabbit polyclonal antibody raised against cytochrome P450a cross-reacted with cytochromes P450b, P450e, and P450f (which are structurally related to cytochrome P450a). The cross-reacting antibodies were removed by passing anti-P450a over an N-octylamino-Sepharose column containing these heterologous antigens. The immunoabsorbed antibody recognized only a single protein (i.e., cytochrome P450a) in liver microsomes from immature male rats treated with Aroclor 1254 (i.e., the microsomes from which cytochrome P450a was purified). However, the immunoabsorbed antibody recognized three proteins in liver microsomes from mature male rats, as determined by Western immunoblot. As expected, one of these proteins (Mr 48,000) corresponded to cytochrome P450a. The other two proteins did not correspond to cytochromes P450b, P450e, or P450f (as might be expected if the antibody were incompletely immunoabsorbed), nor did they correspond t cytochromes P450c, P450d, P450g, P450h, P450i, P450j, P450k, or P450p. One of these proteins was designated cytochrome P450m (Mr approximately 49,000), the other cytochrome P450n (Mr approximately 50,000). Like cytochrome P450a, cytochrome P450n was present in liver microsomes from both male and female rats. However, whereas cytochrome P450a was detectable in liver microsomes from 1-week-old rats, cytochrome P450n was barely detectable until the rats were at least 3 weeks old. Furthermore, in contrast to cytochrome P450a, the levels of cytochrome P450n did not declin appreciably with age in postpubertal male rats. Cytochrome P450m was detectable only in liver microsomes from postpubertal (greater than 4 week-old) male rats. Cytochromes P450m and P450n were isolated from liver microsomes from mature male rats and purified to remove cytochrome P450a When reconstituted with NADPH-cytochrome P450 reductase and lipid, cytochrome P450n exhibited little testosterone hydroxylase activity, whereas cytochrome P450m catalyzed the 15 alpha-, 18-, 6 beta-, and 7 alpha-

hydroxylations of testosterone at 10.8, 4.6, 2.0, and 1.9 nmol/nmol P450/min respectively. The ability of cytochrome P450m to catalyze the 7 alpha-hydroxylation of testosterone was not due to contamination with cytochrome P450a, which catalyzed this reaction at approximately 25 nmol/nmol P450a/min. Cytochrome P450m also converted testosterone to several minor metabolites, including androstenedione and 15 beta-, 14 alpha-, and 16 alpha hydroxytestosterone.(ABSTRACT TRUNCATED AT 400 WORDS)

PMID: 2650624 [PubMed - indexed for MEDLINE]

1: J Biol Chem. 1988 Sep 15;263(26):13310-6.

Bordetella pertussis adenylate cyclase. Identification of multiple forms of the enzyme by antibodies.

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Two forms of Bordetella pertussis adenylate cyclase of 200 and 47 kDa have been purified from dialyzed urea extract of the bacteria to specific activities of 466 and 1685 mumol.min-1.mg-1, respectively. Both forms are activated 50-200-fold by calmodulin. The half-maximum concentration required for th activation of the 200-kDa catalyst is 5.4.10(-9) M, whereas the one required for activation of the 47-kDa catalyst is 1.8.10(-10) M. Polyclonal antibodies raised against the 47-kDa catalyst specifically recognize both forms of the enzyme in purified state as well as in bacterial extracts on immunoblots. The antibody inhibits at similar titer adenylate cyclase activity of the purified forms as well as the activity present in dialyzed urea extract of the bacteria. I also prevents the penetration of the invasive B. pertussis adenylate cyclase into human lymphocytes. The inhibition induced by the antisera is specific tc B. pertussis enzyme, since both calmodulin-dependent brain and sperm adenylate cyclase are not affected by the antibody.

PMID: 2901413 [PubMed - indexed for MEDLINE]

1: Biochem Pharmacol. 1993 Feb 24;45(4):899-907. Related Articles, Linl

Cytochrome P450 isoforms in human fetal tissues related to phenobarbital-inducible forms in the mouse.

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Four polyclonal antibodies raised against purified mouse liver cytochrome P450s representing Cyp1a, Cyp2a, Cyp2b and Cyp2c subfamilies were used to detect their related forms in human adult and fetal tissues. In immunoblot analysis, anti-Cyp2c antibody detected two to three proteins in adult livers and one to three proteins in 70% of the 18 fetal livers studied. Anti-Cyp2a-5 antibody recognized a 50-kDa protein in 50% of the fetal adrenals. Anti-Cyp1a-2 antibody reacted with a single protein (55 kDa) in adult liver. The anti-Cyp2b-10 antibody did not detect proteins in any of the tissues. No proteins were detected in fetal kidneys. There was no coumarin 7-hydroxylas activity (COH) in fetal liver or adrenals. The 7-ethoxycoumarin O-deethylase (ECOD) activities were slightly higher in fetal adrenals (mean 6.1 pmol/mg protein/min) vs livers. The fetal adrenal ECOD activity was not inhibited by the anti-Cyp2a-5 antibody. Aryl hydrocarbon hydroxylase (AHH) activities i fetal livers were about 5% of those in adult livers. AHH activity in fetal liver was not inhibited by the anti-Cyp2c antibody. Testosterone 6 beta-hydroxylase activity was much lower in fetal liver than in adult liver (about 20 and 1700 pmol/mg protein/min, respectively). No immunoinhibition occurred in fetal adrenal progesterone hydroxylation, hepatic benzphetamine N-demethylation and hepatic ethylmorphine N-demethylation. These data suggest that members of the P450 subfamilies 1A, 2A and 2B are expressed a very low level in fetal liver, and that fetal liver may contain members of the 2C subfamily.

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